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Antimicrobial Activity of Combined Plant Extracts of *Ageratum conyzoides* and *Bidens pilosa*

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Gerald Ngo Teke[1*], Flore Nguemaïm Ngoufo[1], Charles Fokunang[2]

[1]Department of Biomedical Sciences, Faculty of Health Sciences, The University of Bamenda, Bambili, Cameroon

[2]Faculty of Medicine and Biomedical Sciences, University of Yaoundé 1, Cameroon

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ABSTRACT

The leaves of *Ageratum conyzoides* and *Bidens pilosa* are known for their antimicrobial activities and used in folkloric medicine. The aim of this study was to evaluate the antimicrobial effects of combined leaf extracts (synergistic, additive or antagonistic) of these plants. The aqueous and methanol leaf extracts of both plants were separately prepared and various extract combinations were evaluated for antimicrobial activities against Gram – and + bacteria and two types of *Candida* fungi. The MICs and MBCs/MFCs for single and combined (1:1 v/v) plant extracts were evaluated at 16, 8, 4, 2, 1 and 0.5 mg/ml using the agar dilution method. Standard antimicrobial agents (ciprofloxacin and miconazole) were used (176 to 5.5 µg/ml). The interaction types for combined plant extracts against microbial growth were determination by finding the fractional inhibitory concentration (FIC). In this study, the MIC values for *B. pilosa* were lower compared to those of *A. conyzoides* both for aqueous and methanol extracts. *Escherichia coli* ATCC28913, *Pseudomonas aeruginosa* ATCC27853 and *Proteus mirabilis* were not sensitive to the aqueous extract of *A. conyzoides*. The FIC indices ranging from 0.5-1 indicated an additive interaction type for the combined extracts against a majority of the studied microorganisms. Indifferent interaction types were observed for the Gram – and Gram + bacteria and yeast. No synergistic or antagonistic interaction type was recorded. Combining the methanol extracts of *Ageratum conyzoides* and *Bidens pilosa* leaves showed additive interaction against some microbial pathogens, suggesting its use as alternative therapy pending further research.

INTRODUCTION

Since time immemorial, plant materials have been used as medicines and as food additives (spices) (Nweze *et al*., 2004). These plants constitute a great reservoir of active chemical compounds that could be used against infectious diseases which accounts for over a third of death cases worldwide (O'Brien, 2004; Leckridge, 2004).In developed countries, it is estimated that 80% of individuals use traditional medicine (Biqiku *et al*., 2016). These medicinal plants represent an alternative treatment in non-severe cases of infectious diseases (Fabricant and Farnsworth, 2001) especially as the pathogenic microbes are gaining resistance against standard antibiotics.

Ageratum conyzoides L.(Asteraceae) is a weed that is erect, annual, branched, slender, hairy and aromatic, growing in the tropics. This herb has been known since ancient times to treat various ailments (burns, wounds, infectious diseases, bacterial infections, arthritis, headaches and dyspnea, pneumonia, inflammatory, asthmatic, spasmodic and haemostatic effects, stomach ailments, gynaecological diseases, leprosy and other skin diseases (Oluwafemi *et al*., 2019). Some researchers have reported the antimicrobial single plant extracts of selected parts or the entire plants (Osezele *et al*., 2013; Oluwafemi *et al*., 2019).

Bidens pilosa L. (Asteraceae) is an annual herb. It is a cosmopolitan weed originally from South America. The ethanol extract of this plant is used in folk medicine as an antihelmintic and protozoacide agent; it also has antiseptic properties. Some yeast and bacteria were said to be sensitive to its polyacetylene extract (Khan *et al*., 2001) and those from different solvents (Falowo and Oladunmoye, 2018; Nyangabo *et al*., 2019). In addition to acetylene, other compounds such as phytosterol, triterpenes and caffeic acid are reported from *Bidens pilosa* (Dagawal and Ghorpade, 2011).

The combination of medicinal plants extracts has been sparingly studied. Most of the preparations of medicinal plants prescribed by local healers and also those available in African local markets contain more than one plant. Commonly available scientific literature is focused on single plant extract antimicrobial activity or plants-antibiotics combinations. Thus, just a few studies have considered combining two or more of different plant extracts in evaluating antimicrobial activities (Biqiku *et al*., 2016).

Hence it was worth to investigate whether the combination of plant extracts (*Ageratum conyzoides* and *Bidens pilosa*) might be synergistic, additive or antagonistic.

MATERIALS AND METHODS

Plant collection

The leaves of two plants namely, *Ageratum conyzoides* L. and *Bidens pilosa* L., which are commonly found, were collected around the campus of the University of Bamenda in May 2019. They were identified by Dr. Tacham Walters, Department of Biological Science, Faculty of Science of The University of Bamenda.

Preparation of methanol and aqueous extracts

Fresh leaves of these plants were separately air dried under shade for three weeks and machine ground into fine powder and separately kept in airtight containers to avoid the absorption of moisture. A quantity of 250 g of the powdered sample was soaked in 2l methanol and water respectively as solvents to extract the bioactive compounds. The containers were appropriately labeled and left for 72 hours (3 days) to macerate while stirring every 6 hours and the process repeated to increase the final extract quantity as needed. Later, it was sieved using cotton wool and then filtered using no. 1 Whatman filter paper. The methanol filtrates were concentrated using rotary evaporator while the aqueous extracts were oven dried at 40°C. Both the methanol and aqueous extracts were separately preserved in a sterile bottle at 4°C until use.

Microorganisms and growth condition

The microorganisms used in this study constituted clinical isolates and standard strains. They included Gram negative bacteria (*Salmonella typhi* ATCC6539, *Klebsiella pneumonia* ATCC2513883, *Escherichia coli* ATCC28913), *Proteus mirabilis*, *Pseudomonas aeruginosa* ATCC27853, *Shigella flexneri*, *Providencia stuartii*, Gram positive bacteria (*Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29212) and two types of fungi (*Candida albicans* (ATCC10231) and *Candida krusei*). The microorganisms were obtained both from the Microbiology/ Bacteriology laboratory of the Yaoundé University Teaching Hospital Center and the Pasteur Centre Research Unit Yaoundé Cameroon. The microorganisms collected were checked for identity and purity using standard biochemical and staining methods (Aneja, 2003). They were later subcultured and maintained on Nutrient agar (NA) in sterile Petri dishes and stored at 4˚C to be used later.

Preparation of microbial suspensions

Microorganisms were sub-cultured prior to each testing in their respective media at 37˚C for 24 hours (bacteria) and 48 hours (yeast). Stock bacterial inoculums suspensions were prepared in sterile normal saline. Three-four well isolated colonies were picked using the inoculating loop and suspended in sterile saline under aseptic conditions. Bacterial suspensions were adjusted visually to 0.5 McFarl and turbidity standards $1x10^8$ CFU/ml while fungal suspensions were adjusted to 1.5×10^6 spores/ml (Teke *et al*., 2013). A quantity of 466 µl of microbial suspension was then pipetted and diluted with 70 ml of 0.9% saline water giving a final microbial load of $1x10^6$ CFU/ml for agar inoculation (Gerald and Betie, 2016; Teke *et al*., 2019).

Susceptibility test

The agar dilution technique was used to test different plant extracts for antimicrobial activities whence the minimum inhibitory concentrations (MICs) and maximum bactericidal and fungicidal concentrations (MBCs and MFCs respectively) were determined in triplicate following CLSI standards (Teke *et al*., 2013; Ballestero-Tellez *et al*., 2017).

Briefly, a concentration of 176 mg/ml was prepared from individual selected plant extracts. A dilution series of single and combined plant extracts ranging from 176 to 5.5 mg/ml and 1 ml of each was carefully and separately transferred into a 20 ml corresponding labeled container. The extracts were combined in a 1:1 v/v ratio; equal volume of each extract was taken and combined to make a working solution. Standard antimicrobial agents (Ciprofloxacin and Miconazole) were used at a range 176 to 5.5 µg/ml. Mueller Hinton and Sabouraud Dextrose agars (for bacteria and yeast respectively) were prepared as recommended by the manufacturer and the sterilized agar allowed to cool to 45˚C in a water-bath. A quantity of 10 ml of molten agar was carefully added to each corresponding container, mixed thoroughly, and poured into prelabeled sterile Petri dishes on the level base surface of the sterile booth. Then the plates were allowed to set at room temperature (30 minutes) and while not fully covered so that no drops of moisture remain on the surface of the agar. A drug-free control plate was equally included for each microbial type. The effective concentrations of plant extracts in the diluted agar were 16, 8, 4, 2, 1 and 0.5 mg/ml.

Now, the inoculating loop was used to transfer a given quantity of microbial suspension onto the agar plates and uniformly seeded by streaking. The susceptibility plates were then placed

in an incubator at 37°C for 24hours (bacteria) and/or 48 hours (yeast). After the incubation period, the agar plates were observed for microbial growth. The MIC corresponded to the lowest test concentration where no growth of microorganism was observed. All tests were performed in triplicates.

For the MBC/MFC, all the test concentrations that inhibited the growth of microorganisms were sorted out and their corresponding freshly prepared agar plate made. A sterile loop was used to collect the seeded microorganism from the MIC assay and transfer by streaking onto the new agar plate and further incubated accordingly. The least concentration that did not show growth of test organisms was considered as the MBC/MFC.

Determination interaction types for combined plant extracts against microbial growth

The methanol leaf extracts of both plants were used since their MIC values were lower, to determine the interaction types of combined extracts (ratio of 1:1 (w/w)) on microbial growth. The extract interactions were achieved by finding the fractional inhibitory concentration (FIC) that were calculated for the 1:1 combinations of the plants per microorganism.

This was determined with the equation below, where (i) and (ii) represented the different 1:1 plant combinations (Mabona *et al*., 2013). The FIC index was expressed as the sum of FIC (i) and FIC (ii) and this was used to classify the interaction as either synergistic (≤0.50), additive (0.50–1.00), indifferent (>1.00–4.00) or antagonistic (>4.00) (Teke *et al*., 2019).

$$\text{FIC(i)} = \frac{\text{MIC of plant extract1 in combination with plant extract2}}{\text{MIC of plant extract1 independently}}$$

$$\text{FIC(ii)} = \frac{\text{MIC of plant extract2 in combination with plant extract1}}{\text{MIC of plant extract2 independently}}$$

RESULTS

Antimicrobial activity of individual and combined plant extracts

In this study, the antimicrobial activity of both individual and combined plant extracts was conducted and the MICs and MBCs/MFCs obtained are displayed as indicated in Table No. 1. Generally, the MIC values for *B. pilosa* were lower compared to those of *A. conyzoides* both for aqueous and methanol extracts. *Escherichia coli* ATCC28913, *Pseudomonas*

aeruginosa ATCC 27853and *Proteus mirabilis* were not sensitive to the aqueous extract of *A. conyzoides*. In some cases for the combined extracts, the MIC values were lower compared to those of individual plant extracts. However, the antibacterial activity of this combination did not improve from the independent MIC values for some of the microorganisms. All tested fungi were sensitive to the plant extracts.

Table No. 1: MIC and MBC/ MFC values (mg/ml) **for *A. conyzoides* and *B. pilosa* single and combined leaf extracts on microorganisms**

Microorganisms	*A. conyzoides*		*B. pilosa*		Combined plants		References
	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Ciprofloxacin and Miconazole (µg/ml)
Escherichia coli ATCC28913	NA	8/16	2/4	1/2	2/4	0.5/1	1/2
Pseudomonas aeruginosa ATCC27853	NA	NA	4/8	4/8	4/8	2/4	2/8
Klebsiella pneumonia ATCC2513883	16/>16	8/16	2/4	1/2	2/4	1/2	2/4
Salmonella typhi ATCC6539	16/>16	8/>16	2/4	1/2	2/4	1/2	2/8
Shigella flexneri	16/>16	8/16	4/8	2/4	4/8	1/2	4/8
Proteus mirabilis	NA	4/8	4/8	2/4	4/8	2/4	2/4
Staphylococcus aureus ATCC29213	16/>16	8/16	2/4	1/2	2/4	1/2	4/16
Enterococcus faecalis, ATCC29212	16/>16	4/8	4/8	2/4	2/4	1/2	1/4
Providencia stuartii	16/16	4/8	4/8	4/8	4/8	2/4	2/4
Candida albicans	4/8	2/4	2/4	2/4	2/4	1/2	4/8
Candida krusei	4/8	1/2	2/4	0.5/1	2/4	0.5/1	2/8

Interaction types for combined plant extracts against microbial growth

The MIC values of the methanol extracts for both plants (lower values) were further subjected to interaction type analysis. The FIC indices ranging from 0.5-1 indicated an additive interaction type for the combined extracts against a majority of the studied microorganisms (Table No. 2). Indifferent interaction types were observed for the Gram + and Gram – bacteria and yeast. No synergistic or antagonistic interaction type was recorded.

Table No. 2: MIC values, FIC indices and interaction types for *A. conyzoides* and *B. pilosa* combined leaf extracts on microorganisms

Microorganism	*A. conyzoides*(A)+ *B. pilosa*(B) (independent MIC, mg/ml)	MIC values in mg/mL (combination)	FIC(i)/ FIC(ii)	FIC index	Interaction type
Escherichia coli ATCC28913	A (8) + B(1)	0.5	0.0625/0.5	0.5625	Additive
Pseudomonas aeruginosa ATCC27853	A (0) + B(4)	2	0/0.5	0.5	Additive
Klebsiella pneumonia ATCC2513883	A (8) + B(1)	1	0.125/1	1.125	Indifferent
Salmonella typhi ATCC6539	A (8) + B(1)	1	0.125/1	1.125	Indifferent
Shigella flexneri	A (8) + B(2)	1	0.125/0.5	0.625	Additive
Proteus mirabilis	A (4) + B(2)	2	0.5/1	1.5	Indifferent
Staphylococcus aureus ATCC29213	A (8) + B (1)	1	0.125/1	1.125	Indifferent
Enterococcus faecalis, ATCC29212	A (4) + B (2)	1	0.25/0.5	0.75	Additive
Providencia stuartii	A (4) + B (4)	2	0.5/0.5	1	Additive
Candida albicans	A (2) + B (2)	1	0.5/0.5	1	Additive
Candida krusei	A (1) + B (0.5)	0.5	0.5/1	1.5	Indifferent

Interaction as either synergistic (≤0.50), additive (0.50–1.00), indifferent (>1.00–4.00) or antagonistic (> 4.00)

DISCUSSION

Infectious diseases are known to accounts for over a third of death cases worldwide (O'Brien, 2004; Leckridge, 2004). These infectious microorganisms are gaining resistance against available standard drugs, constituting an important public health problem, especially for the vulnerable populations (WHO, 2014). Hence the search for therapeutic alternatives among traditionally used medicinal plants since these plants are known to harbor a vast majority of active secondary metabolites (Fabricant and Farnsworth, 2001).

In this study, the aqueous and methanol plant extracts were active, inhibiting microbial growth.The methanol leaf extracts of both *A. conyzoides* and *B. pilosa* showed stronger activity against the bacteria and fungi studied. This corroborates with the findings of Ijeh *et al*. (2006) and Osezele *et al*. (2013) indicating that methanol extracts were the most potent of all the extracts harboring a majority of highly non polar compound active component. Moreover, plant extracts in organic solvents like methanol are known to show consistent antimicrobial activity when compared to water extract. Methanol extracts possess the ability to easily dissolve and diffuse in wide variety of media (Nair *et al*., 2005). This suggests that active secondary metabolites present in these plant extracts are capable of counteracting microbial activity (Bartolome *et al*., 2013). Some researchers have equally reported on the antibacterial and antifungal potentials of *A. conyzoides* (Osezele *et al*., 2013) and *B. pilosa* (Shandukani *et al*., 2018; Nyangabo *et al*., 2019).

Our findings are in concurrence to the reports of Dayie *et al*. (2008), Garg (2015) and Singh *et al*. (2016) for *A. conyzoides* extracts being more effective against *Staphylococcus* strains than *E. coli* and *Pseudomonas* strains.

The bacterial activities for the aqueous extract of *B. pilosa* in this study were comparable to those reported by Lawal *et al*. (2015) against similar bacteria. Equally, Osezele *et al*. (2013) reported higher MIC values (50-200 mg/ml) for both aqueous and methanol extracts. These variations could be due to differences in geographic factors in area of plant growth and collection; microbial strength in developing resistance and the laboratory technique used in the evaluation (Agem *et al*., 2015). It was observed that the MIC values were lower compared to the MBC values. This suggests that these extracts were bacteriostatic at lower concentrations and bactericidal at higher concentrations.

A good number of active compounds have been isolated from *A. conyzoides* (Gunawan *et al*., 2008) and *B. pilosa* (Wen-Chin *et al*., 2019).

Combined extract activity

Traditional practitioners usually combine several plant species when preparing herbal drugs (Van and Viljoen, 2011). It is understood that the combined extracts or drugs used in the treatment of ailments (through synergism interaction) will improve the efficacy, minimize toxicity, cure faster compared to individual extracts. Moreover, resistant microbes are better treated and a broader spectrum of anti-infective agent observed than using just a monotherapy (Van and Viljoen, 2011; Rubaka *et al*., 2014). Most of the reports on combination of natural products and evaluating of antimicrobial activities found in the literature were centered on plant extract combined with conventional antibacterials and/or antifungals (Dayang and Vimashiinee, 2016; Danielle *et al*., 2019). Just a few scientific reports were gotten on the combination of different plant extracts or extracts of parts of the same plant (Bakarnga *et al*., 2016; Shandukani *et al*., 2018).

In this paper, the interaction type of combining two extracts, *A. conyzoides* and *B. pilosa*, on some bacteria and fungi were evaluated. A variation among additive interaction (FIC index of 0.50–1.00) and indifferent effect of interaction (FIC index of >1.00–4.00) was observed across the Gram - and + bacteria and the fungi studied. Based on the FIC indices, a similar plant extract interaction type results of been indifferent was reported by Shandukani *et al*. (2018) for *B. pilosa* plus *Dichrostachys cinerea* extracts against the growth of *K. pneumonia, E. coli, S. typhimurium*. Moreover, a combination of plant extracts could lead to promising synergistic antibacterial effects as exerted against *Escherichia coli* (Bakarnga *et al*., 2016). Additive interaction of plant extracts and/or have been appreciated as an increase in efficacy is obtained compared to individual treatments (Sandeep *et al*., 2016).

Some researchers have suggested different mechanisms of interactions of compounds in combined preparations and that they are conditioned by several factors (Szalek *et al*., 2006).

It is worth mentioning that combinations of antimicrobial agents that show synergistic and/or additivity interactions could potentially improve clinical outcome of patient suffering from infections that are difficult to treat (Dayang and Vimashiinee, 2016).

CONCLUSION

The combination of methanol extracts from *Ageratum conyzoides* and *Bidens pilosa* leaves showed additive interactions against microbial pathogens studied (Gram - and + bacteria and *C. albicans)*, suggesting its use over single plant extract as alternative therapy pending further research.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

GNT conceived the study and designed the methods. GNT and FNN did the experimental and laboratory work, collected and transported data and other materials. GNT drafted the manuscript. CF edited and finalized the manuscript for publication. All authors read and approved the final manuscript.

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